

Spotting the target: microarrays for disease gene discovery

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Microarray technologies enable genome-scale expression measurements. Already proved to be of value for the functional analysis of individual genes and biological processes, the application of expression profiling to disease gene discovery is now growing in importance and practicality.

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Abbreviations

CGH comparative genomic hybridization
NF2 neurofibromatosis type 2
PARP poly(ADP-ribose) polymerase
TD Tangle disease

Introduction

Positional cloning projects have been greatly facilitated by the availability of increasingly precise maps and sequence databases for diverse species. This same avalanche of genomic data has inspired an intense effort to study aspects of genome function in a high-throughput fashion. The parallel analysis of gene expression has emerged as one of the most productive embodiments of this approach.

Practical technologies for large-scale gene-expression analysis are now being widely implemented. Microarrays comprising either oligonucleotides or cDNA fragments representing thousands of genes are well suited to the analysis of multiple samples [1,2]. To obtain genome-scale expression data, mRNA from the source of interest is converted to an appropriately labeled form and hybridized to the microarray. Both radioactive and fluorescence-detection strategies are in use to measure the resulting hybridization signal. The resulting raw data — an image obtained from a fluorescence scanner or phosphorimager — is processed with computer software to generate a spreadsheet of gene-expression values. The application of statistical techniques to microarray data allows classification and class discovery within a group of samples, and clustering of genes according to their pattern of expression.

Microarrays have been successfully applied to characterize biological processes and to dissect pathways downstream of a particular gene of interest. Studies in the yeast *Saccharomyces cerevisiae*, with its relatively small genome and highly tractable genetics, have led the way and continue with recent reports on signal transduction [3], meiosis [4] and transcript localization [5]. Despite the challenges posed by their genome sizes, large-scale expression analysis in mammals is also becoming increasingly productive.

As the technology for microarray analysis has matured and disseminated, new applications continue to be developed. One frequently discussed area is the potential use of microarray expression analysis in projects to positionally clone and discover disease genes. Although reviews of this topic outnumber reports of concrete achievement, it is appropriate to examine the state of the art and to consider how microarray analysis might accelerate these types of research. I discuss these points, together with recent developments in microarray research, in this review.

How might microarrays help find hereditary disease genes?

Several major approaches to locating hereditary disease genes might be imagined. In the simplest case, the target gene of interest might be identified directly by characteristic changes in expression level across a series of samples. Alternatively, statistical analysis of microarray data might aid gene discovery by revealing pathways related to the target gene and facilitating identification of candidate genes.

Microarrays can also be used to analyze genomic DNA rather than mRNA. This is illustrated by the special case of copy-number change in cancer, where it is possible to use array-format comparative genomic hybridization (CGH) to define genes associated with cancer progression [6*,7,8*]. In CGH, gene copy number is measured in a DNA sample labeled with one fluorochrome by comparison to the signal obtained by simultaneous hybridization of normal DNA labeled with a second fluorochrome. In principle, copy-number data can be linked to expression data to define a list of candidate target genes associated with gain of chromosomal regions [9*,10]. Although there is no example to date, tumor suppressors might be mapped by linking loss of gene expression to regions of deletion in tumors.

Of course, microarrays can be used as sophisticated dot blots to screen arrays of clones isolated with techniques such as RDA [11]. (RDA [representational difference analysis] is a PCR-based subtraction technique that can be used to isolate DNA fragments that vary in abundance between two sources.) Stephan *et al.* [12] have identified exons of the Niemann-Pick Type C disease isolated from arrayed genomic sequences using mRNA from cells differentially expressing *NPC1*. Finally, genes might be linked to specific phenotypes, particularly in yeast, through methods that allow genome-wide mutational screens using microarrays as a readout [13].

Finding the best candidate

It is enticing to hope that analysis of microarray data might lead to the direct identification of disease genes. Ideally, one would compare a group of samples of varying genotype and identify good candidate genes by their pattern of gene

expression. The expected signature of a mutant gene is reduced expression level in samples with the abnormal allele. For this strategy to work, the mutant allele would have to be either deleted or result in a poorly expressed transcript.

Fortunately, the phenomenon of nonsense-mediated decay of mRNA gives some reason to hope that this result might actually be achieved. Nonsense-mediated decay (reviewed in [14]) results in the degradation of certain mRNAs containing premature termination codons. This phenomenon has been observed in a number of disease genes [15,16].

In addition, abnormalities in 3'-untranslated region structure that interfere with normal polyadenylation may also lead to reduced survival of transcripts [17]. A reduction in steady-state mRNA levels of disease genes cannot be assumed, however, because the competence of a transcript to undergo nonsense-mediated decay is variable and some mutations may result in exon skipping [18,19], as has been shown by Liu *et al.* [20] for the *BRCA1* gene. This strategy also requires a sufficient number of samples from cells or tissues affected by the disease to help optimize the downstream data analysis.

Although obvious, an additional requirement of expression-based strategies is that the target gene is actually represented on the microarrays used. Although arrays of more than 10,000 genes are commonplace and complete genome microarrays can be anticipated, they are not yet routinely available. It is also probably unrealistic to assume that only a single gene or a few genes will stand out from the crowd with sufficient clarity to allow easy candidate selection. More likely, a strategy combining positional information with expression information will be necessary.

This combination of approaches has been used by Lawn *et al.* [21**] in the discovery of the Tangier disease (TD) gene *ABCI*. Microarray analysis led to the generation of a list of 175 cDNAs underexpressed by 2.5-fold or more in the fibroblasts of an affected individual. By combining this data with linkage information that localized the disease gene to chromosome 9q between the markers W1-14706 and W1-4062, the candidate list was narrowed sufficiently to identify the gene *ABCI*, which did indeed carry mutations.

Notably, Lawn *et al.* [21**] used commercial cDNA arrays containing 58,800 cDNAs, which presumably provided a reasonably thorough genome scan. One might imagine that regional searches could be made by constructing targeted microarrays covering a particular candidate region. This has been done for the X chromosome and for chromosome 17q [9*,22**].

It is important to bear in mind that almost all research employing microarray expression analysis depends heavily on statistical analysis to extract the most useful information from the huge number of data points generated. This means

that any investigator attempting to use microarrays for disease gene discovery will also seek to go beyond this direct type of search and also examine the broader effects of mutation on gene expression in samples from affected individuals.

If one were not able to identify easily a candidate gene by virtue of its underexpression, perhaps the recognition of pathways altered consistently across a set of specimens might lead to the identification of good candidate genes or, at the very least, might illuminate some aspects of pathogenesis.

Finding the disease pathway affected by known genes

The complexity of microarray data is illustrated by another interesting feature of the TD data — the overexpression of 375 cDNAs by 2.5-fold or more. This result, revealing a total of 550 cDNAs with altered expression, is probably typical of what might be expected in most projects. In addition to innumerable technical factors, variations in gene expression across samples might be due to random fluctuations or confounding variables such as age, sex, site of sample and irrelevant genetic variations. Still, it would seem reasonable to suppose that the presence of a mutation in a pathway might frequently lead to secondary events affecting the level of expression of many other genes functionally connected to the disease gene.

Most published examples attempting to place genes from microarray data on samples carrying mutations into coherent pathways are in the setting of model systems for which the mutation is already known. McNeish *et al.* [23*] have examined a mouse model of TD with microarrays containing 11,000 genes and have identified 131 genes with greater than 1.8-fold differential regulation, many of which can be grouped into a few function-related categories. Their study demonstrates how studies of a relatively tractable experimental model can enhance the value of data obtained from human samples.

Likewise, Soukas *et al.* [24*] examined gene expression in white adipose tissue from mice expressing varying levels of the leptin gene. Seventy-seven genes were dysregulated by threefold or more in these *ob/ob* mice, including a number of key genes in fat metabolism. One cluster of genes was coordinately regulated by SREBP-1/ADD1, but the regulating mechanisms linking genes in several other clusters remain unknown. Although the complete pattern of changes observed cannot be explained as yet, the relevance of the leptin gene to fat metabolism is amply demonstrated.

Simbulan-Rosenthal *et al.* [25*] examined fibroblasts from mice deficient in poly(ADP-ribose) polymerase (PARP) with microarrays covering 11,000 genes and identified 91 genes differentially regulated by at least twofold relative to wild-type fibroblasts. About 40% of these could be related to either the cell cycle or remodeling of the cytoskeleton or extracellular matrix — processes known to be associated with PARP function.

Callow *et al.* [26] examined livers from apolipoprotein A1 knockout mice, scavenger receptor B1 transgenic mice and wild-type mice on microarrays containing 5600 cDNAs. They used *t*-test statistics to identify a small number of genes that differed significantly across these conditions.

For disease gene discovery, the interpretation of expression data in terms of pathways is more difficult because there is no *a priori* knowledge of the disease gene function. This leads to a consideration of the process of grouping differentially expressed genes into pathways.

Placing genes in pathways to gain clues about unknown genes

Can pathways actually be discerned from microarray data? It is worthwhile considering some of the individual steps in the process of deducing pathway information from these data. Clustering of genes into co-regulated groups is computationally straightforward and readily generates this type of information [27]. Similarly, there has been great success in classifying biological samples from microarray data, particularly for cancer specimens [28,29,30,31,32]. These studies are promising in identifying critical genes for cancer progression at the expression level, although these are not necessarily 'disease genes' in the genetic sense [33].

Nonetheless, Hedenfalk *et al.* [34] have even shown that it is possible to sort breast cancer specimens according to the presence of hereditary mutations in *BRCA1* or *BRCA2*. One of the most striking results in their study was the demonstration that a sample that clustered with those from patients carrying mutations in *BRCA1* lacked a *BRCA1* mutation but was highly methylated at the *BRCA1* promoter.

It might be hoped that this approach could aid complex disease gene discovery by sorting samples into groups that share a common genetic defect. When combined with positional data from linkage analysis, such an approach might be expected to take on a significant role in the study of complex disease.

In contrast to clustering samples and genes, the interpretation of expression data to infer the pathway affected by a disease gene mutation is much more problematic. The initial problem one faces in this type of analysis is the limited annotation of the genome. When examining an expression database, one immediately encounters difficulty in placing genes into functional categories. This is beset with a number of obstacles, the first of which are the numerous aliases that confuse gene nomenclature.

The introduction of two on-line resources, LocusLink and Refseq, have gone a long way towards overcoming this problem by providing a unique identifier and curated sequence for each gene [35]. This is absolutely critical to the next phase of analysis, which is the cross-reference to other databases of gene function including, most importantly, literature databases. Frequently, different functions or interpretations

of gene function are linked to distinct aliases for a given gene. Only by thoroughly combing the literature, can the most comprehensive picture of gene function be obtained. Substantial efforts are being made to organize the genes of known function into meaningful categories.

Although a detailed discussion of the problem of gene annotation is beyond the scope of this review, the public availability of certain resources should be noted. In particular, the Gene Ontology consortium uses a common language to organize functional information in all species [36]. Currently, the Gene Ontology database contains database links for *Drosophila*, *S. cerevisiae*, mouse and *Caenorhabditis elegans*. Genes are categorized in three hierarchical schemes according to molecular function, biological process and cellular component.

Methods to process groups of genes with respect to literature databases are also under development [37–39]. One system, High-density Array Pattern Interpreter (HAPI; <http://array.ucsd.edu/hapi/>), is publicly available. It is anticipated that search engines that can carry out these computations with the output of expression databases will significantly accelerate the process of organizing data from microarrays.

Although it is relatively straightforward to identify lists of genes that are co-regulated across a set of samples, this may not be a sufficiently sensitive method to extract functionally related genes. Intensive efforts to establish alternate computational methods are continuing.

Seungchan *et al.* [40] have described a multivariate technique that has the potential to identify relationships among genes that are refractory to methods based on linear correlation. Akutsu *et al.* [41] have proposed a method for modeling gene expression in terms of Boolean networks, whereas Friedman *et al.* [42] have proposed a Bayesian method. Hastie *et al.* [43] have described a method termed 'gene shaving', which differs from hierarchical clustering in that genes may belong to more than one cluster. Brown *et al.* [44] have advocated the use of method based on the theory of 'support vector machines', a computer learning method that they have adapted to the functional categorization of expression data.

Finding regulatory motifs

One great challenge remaining in the analysis of mammalian expression data will be to link this information to regulatory elements in the genome sequence. Promising results in yeast continue to appear. Iyer *et al.* [45] have taken advantage of the small size of the yeast genome to array non-coding DNA and identify the genes regulated by the cell-cycle transcription factors SBF and MBF. Ren *et al.* [46] have achieved similar results for Gal4 and Ste12, and Livesey *et al.* [47] have identified the response element configuration and genes responsive to the mouse homeobox gene *Crx*.

The development of progressively more sophisticated computational methods increases optimism that genes related to a phenotype can be accurately extracted and placed in functionally related groups to help generate new hypotheses. Even with this goal accomplished, one would expect that the effects of mutation on one biochemical pathway will radiate to affect numerous other pathways. Identifying the pathway primarily affected will be a significant challenge.

Using microarrays to map genomic DNA

Although using microarrays to identify regions of copy-number change in cancers has received the most attention, array format CGH might also be useful for mapping hereditary disease genes. Bruder *et al.* [48**] have used microarrays tiled across a 7-Mb region including the neurofibromatosis type 2 gene (NF2) to analyze DNA from 116 NF2 patients. Using this exquisitely accurate system, they were able to identify 24 patients with gene deletions and show that there was no correlation with disease severity.

In principle, this type of approach could be applied to a region containing an unknown disease gene. Because positional cloners frequently assemble contigs covering regions of linkage, the availability of genomic clones may not be problematic. However, the technology for arraying and accurately determining copy number in this setting is still confined to a few laboratories.

Conclusions

Unquestionably, large-scale expression analysis is now established in the study of genome function. The power of this approach continues to be enhanced by technical advances and, importantly, by the development of very large coherent expression databases from samples collected across a broad range of conditions [49**]. The recent report from Shoemaker *et al.* [50] points to the future with microarrays composed of over one million oligonucleotides representing 442,785 exons predicted from the draft human genome sequence. These developments suggest that microarray analysis will increasingly merit consideration as an ancillary technique to facilitate hereditary disease gene discovery.

Update

Loftus and Pavan have recently used melanocyte-specific microarrays to identify a mouse coat color gene (S Loftus, W Pavan, personal communication).

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